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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **GC381-US**

U.S. APPLICATION NO. (If known, see 37 CFR 1.5) [please provide]: 09/462846

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/14529	14 July 1998	15 July 1997

TITLE OF THE INVENTION
Proteases from Gram-Positive Organisms

APPLICANT(S) FOR DOE/EO/US

David A. ESTELL, US Citizen, residing in San Mateo, California 94403, USA

Applicant herewith submits to the United States Designated/Elected Office (do/eo/us) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT submission of items concerning a filing under 35 U.S.C.** 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor/s (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16 below concern document/s or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary Amendment
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    - Copy if IPER
    - Copy of Publication of the International Search Report

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) [please provide] 09/462846	INTERNATIONAL APPLICATION NO. PCT/US98/14529	ATTORNEY'S DOCKET NUMBER GC381-US

17. ☒ The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492(2)(1)-(5)):

Search Report has been prepared by the EPO or JPO	$ 840
International preliminary examination fee paid to USPTO (37 CFR 1.482)	$ 96
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(2)(2))	$
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(2)(2)) paid to USPTO	$
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$

ENTER APPROPRIATE BASIC FEE AMOUNT =					$ 936
Surcharge of $130 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(4)).					$
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE	
Total Claims	21	- 20 =	1	x $18.00	$ 18
Independent claims	7	- 3 =	4	x $78.00	$ 312
MULTIPLE DEPENDENT CLAIM(S) (IF APPLICABLE)				+ $250.00	$ 250
TOTAL OF ABOVE CALCULATIONS =					$1516
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28)					
SUBTOTAL =					$1516
Processing fee of $130 for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+	$
TOTAL NATIONAL FEE =					$1516
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property					$ 40
TOTAL FEES ENCLOSED =					$1556
				Amount to be refunded:	$
				charged:	$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **07-1048** in the amount of $1556 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **07-1048**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

In re Patent Application of David A. ESTELL US Serial No. [please provide] Based on PCT/US98/14529, filed 14 July 1998 For: **Proteases from Gram-Positive Organisms**	TRANSMITTAL OF PATENT APPLICATION UNDER 35 USC 371

I hereby certify that this paper and attachments are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicate hereunder and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C 20231

13 January 2000

By: _____
Anke E. Luttrell

BOX PCT
Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

Attached, for entering the national phase of International Patent Application PCT/US98/14529, are the following documents:

1. Form PTO 1390 - Transmittal Letter to the US DO/EO/US Concerning a Filing under 35 USC 371;
2. International Patent Application PCT/US98/14529
3. International Search Report
4. Original Declaration/Power of Attorney
5. Original Assignment with Recordation Coversheet.

Respectfully submitted,

GENENCOR INTERNATIONAL, INC.

Date: 10 Jan. 2000

By: _____
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GC381-US

PROTEASES FROM GRAM-POSITIVE ORGANISMS

FIELD OF THE INVENTION

The present invention relates to cysteine proteases derived from gram-positive microorganisms. The present invention provides nucleic acid and amino acid sequences of cysteine protease 1, 2 and 3 identified in *Bacillus*. The present invention also provides methods for the production of cysteine protease 1, 2 and 3 in host cells as well as the production of heterologous proteins in a host cell having a mutation or deletion of part or all of at least one of the cysteine proteases of the present invention.

BACKGROUND OF THE INVENTION

Gram-positive microorganisms, such as members of the group *Bacillus*, have been used for large-scale industrial fermentation due, in part, to their ability to secrete their fermentation products into the culture media. In gram-positive bacteria, secreted proteins are exported across a cell membrane and a cell wall, and then are subsequently released into the external media usually maintaining their native conformation.

Various gram-positive microorganisms are known to secrete extracellular and/or intracellular protease at some stage in their life cycles. Many proteases are produced in large quantities for industrial purposes. A negative aspect of the presence of proteases in gram-positive organisms is their contribution to the overall degradation of secreted heterologous or foreign proteins.

The classification of proteases found in microorganisms is based on their catalytic mechanism which results in four groups: the serine proteases; metalloproteases; cysteine proteases; and aspartic proteases. These categories can be distinguished by their sensitivity to various inhibitors. For example, the serine proteases are inhibited by phenylmethylsulfonylfluoride (PMSF) and diisopropylfluorophosphate (DIFP); the metalloproteases by chelating agents; the cysteine enzymes by iodoacetamide and heavy metals and the aspartic proteases by pepstatin. The serine proteases have alkaline pH optima, the metalloproteases are optimally active around neutrality, and the cysteine and aspartic enzymes have acidic pH optima (Biotechnology Handbooks, *Bacillus*. vol. 2, edited by Harwood, 1989 Plenum Press, New York).

The activity of cysteine protease depends on a catalytic dyad of cysteine and histidine with the order differing among families. The best known family of cysteine proteases is that of papain having catalytic residues Cys-25 and His-159. Cysteine proteases of the papain family catalyze the hydrolysis of peptide, amide, ester, thiol ester and thiono ester bonds. Naturally occurring inhibitors of cysteine proteases of the papain family are those of the cystatin family (Methods in Enzymology, vol. 244, Academic Press, Inc. 1994).

SUMMARY OF THE INVENTION

The present invention relates to the unexpected and surprising discovery of three heretofore unknown or unrecognized cysteine proteases found in *Bacillus subtilis*, designated herein as CP1, CP2 and CP3, having the nucleic acid and amino acid as shown in Figures 1A-1B, Figures 5A-5B and 6A-6B, respectively. The present invention is based, in part, upon the presence of the characteristic cysteine protease amino acid motif GXCWAF found in uncharacterised translated genomic nucleic acid sequences of *Bacillus subtilis*. The present invention is also based in part upon the structural relatedness that CP1 has with the cysteine protease papain specifically with respect to the location of the catalytic histidine/alanine and asparagine/serine residues and the structural relatedness that CP1 has with CP2 and CP3.

The present invention provides isolated polynucleotide and amino acid sequences for CP1, CP2 and CP3. Due to the degeneracy of the genetic code, the present invention encompasses any nucleic acid sequence that encodes the CP1, CP2 and CP3 amino acid sequence shown in the Figures.

The present invention encompasses amino acid variations of *B.subtilis* CP1, CP2 and CP3 amino acids disclosed herein that have proteolytic activity. *B. subtilis* CP1, CP2 and CP3, as well as proteolytically active amino acid variations thereof, have application in cleaning compositions. In one aspect of the present invention, CP1, CP2 or CP3 obtainable from a gram-positive microorganism is produced on an industrial fermentation scale in a microbial host expression system. In another aspect, isolated and purified recombinant CP1, CP2 or CP3 obtainable from a gram-positive microorganism is used in compositions of matter intended for cleaning purposes, such as detergents. Accordingly, the present invention provides a cleaning composition comprising at least one of CP1, CP2 and CP3 obtainable from a gram-positive microorganism. The cysteine protease may be used alone in the cleaning composition or in combination with other enzymes and/or mediators or enhancers.

The production of desired heterologous proteins or polypeptides in gram-positive microorganisms may be hindered by the presence of one or more proteases which degrade the produced heterologous protein or polypeptide. Therefore, the present invention also encompasses gram-positive microorganism having a mutation or deletion of part or all of the gene encoding CP1 and/or CP2 and/or CP3, which results in the inactivation of the CP1 and/or CP2 and/or CP3 proteolytic activity, either alone or in combination with deletions or mutations in other proteases, such as apr, npr, epr, mpr for example, or other proteases known to those of skill in the art. In one embodiment of the present invention, the gram-positive organism is a member of the genus *Bacillus*. In another embodiment, the *Bacillus* is *Bacillus subtilis*.

In another aspect, the gram-positive microorganism host having one or more deletions or mutations in a cysteine protease of the present invention is further genetically

engineered to produce a desired protein. In one embodiment of the present invention, the desired protein is heterologous to the gram-positive host cell. In another embodiment, the desired protein is homologous to the host cell. The present invention encompasses a gram-positive host cell having a deletion or interruption of the naturally occurring nucleic acid encoding the homologous protein, such as a protease, and having nucleic acid encoding the homologous protein or a variant thereof re-introduced in a recombinant form. In another embodiment, the host cell produces the homologous protein. Accordingly, the present invention also provides methods and expression systems for reducing degradation of heterologous or homologous proteins produced in gram-positive microorganisms comprising the steps of obtaining a *Bacillus* host cell comprising nucleic acid encoding said heterologous protein wherein said host cell contains a mutation or deletion in at least one of the genes encoding cysteine protease 1, cysteine protease 2 and cysteine protease 3; and growing said *Bacillus* host cell under conditions suitable for the expression of said heterologous protein. The gram-positive microorganism may be normally sporulating or non-sporulating.

The present invention provides methods for detecting gram positive microorganism homologs of *B. subtilis* CP1, CP2 and CP3 that comprises hybridizing part or all of the nucleic acid encoding *B. subtilis* CP1, CP2 and CP3 with nucleic acid derived from gram-positive organisms, either of genomic or cDNA origin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-1B shows the DNA (SEQ ID NO:1) and amino acid sequence for CP1 (YJDE) (SEQ ID NO:2).

Figure 2 shows an amino acid alignment with papain (SEQ ID NO:3) (accession number papa_carpa.p) with the cysteine protease CP1, designated YJDE. For Figures 2, 3 and 4, the motif GXCWAF has been marked along with the catalytic cysteine and the conserved catalytic histidine/alanine and asparagine/serine residues.

Figure 3 shows amino acid alignment of CP1 (YJDE) (SEQ ID NO:2) with CP3 (PMI) (SEQ ID NO:5).

Figure 4 shows the amino acid alignment of CP1 (YJDE) (SEQ ID NO:2) with CP2 (YdhS).

Figure 5A-5B shows the amino acid (SEQ ID NO:6) and nucleic acid sequence for CP2 (YdhS) (SEQ ID NO:7).

Figure 6A-6B shows the amino acid (SEQ ID NO:4) and nucleic acid sequence for CP3 (PMI) (SEQ ID NO:5).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

As used herein, the genus *Bacillus* includes all members known to those of skill in the art, including but not limited to *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. ciculans, B. lautus and B. thuringiensis.*

The present invention relates to novel CP1, CP2 and CP3 from gram positive organisms. In a preferred embodiment, the gram-positive organisms is a *Bacillus*. In another preferred embodiment, the gram-positive organism is *Bacillus subtilis*. As used herein, "*B.subtilis* CP1, CP2 or CP3" refers to the amino acid sequences shown in Figures. Figures 1A-1B show the amino acid and nucleic acid seqeunce for CP1 (YJDE); Figures 5A-5B show the amino acid and nucleic acid sequence for CP2 (YDHS); and Figures 6A-6B show the amino acid and nucleic acid sequences for CP3 (PMI). The present invention encompasses amino acid variations of the amino acid sequences disclosed in Figures 1A-1B and 5A-5B and 6A-6B that have proteolytic activity. Such proteolytic amino acid variants can be used in cleaning compositions.

As used herein, "nucleic acid" refers to a nucleotide or polynucleotide sequence, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be double-stranded or single-stranded, whether representing the sense or antisense strand. As used herein "amino acid" refers to peptide or protein sequences or portions thereof. A "polynucleotide homolog" as used herein refers to a gram-positive microorganism polynucleotide that has at least 80%, at least 90% and at least 95% identity to *B.subtilis* CP1, CP2 or CP3, or which is capable of hybridizing to *B.subtilis* CP1, CP2 or CP3 under conditions of high stringency and which encodes an amino acid sequence having cysteine protease activity.

The terms "isolated" or "purified" as used herein refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

As used herein, the term "heterologous protein" refers to a protein or polypeptide that does not naturally occur in a gram-positive host cell. Examples of heterologous proteins include enzymes such as hydrolases including proteases, cellulases, amylases, carbohydrases, and lipases; isomerases such as racemases, epimerases, tautomerases, or mutases; transferases, kinases and phophatases. The heterologous gene may encode therapeutically significant proteins or peptides, such as growth factors, cytokines, ligands, receptors and inhibitors, as well as vaccines and antibodies. The gene may encode commercially important industrial proteins or peptides, such as proteases, carbohydrases such as amylases and glucoamylases, cellulases, oxidases and lipases. The gene of interest may be a naturally occurring gene, a mutated gene or a synthetic gene.

The term "homologous protein" refers to a protein or polypeptide native or naturally occurring in a gram-positive host cell. The invention includes host cells producing the

homologous protein via recombinant DNA technology. The present invention encompasses a gram-positive host cell having a deletion or interruption of naturally occurring nucleic acid encoding the homologous protein, such as a protease, and having nucleic acid encoding the homologous protein, or a variant thereof, re-introduced in a recombinant form. In another embodiment, the host cell produces the homologous protein.

As used herein, the term "overexpressing" when refering to the production of a protein in a host cell means that the protein is produced in greater amounts than its production in its naturally occurring environment.

As used herein, the phrase "proteolytic activity" refers to a protein that is able to hydrolyze a peptide bond. Enzymes having proteolytic activity are described in Enzyme Nomenclature, 1992, edited Webb Academic Press, Inc.

Detailed Description of the Preferred Embodiments

The unexpected discovery of the cysteine proteases CP1, CP2 and CP3 in *B.subtilis* provides a basis for producing host cells, expression methods and systems which can be used to prevent the degradation of recombinantly produced heterologous proteins. In a preferred embodiment, the host cell is a gram-positive host cell that has a deletion or mutation in the naturally occurring cysteine protease said mutation resulting in deletion or inactivation of the production by the host cell of the proteolytic cysteine protease gene product. The host cell may additionally be genetically engineered to produced a desired protein or polypeptide.

It may also be desired to genetically engineer host cells of any type to produce a gram-positive cysteine protease. Such host cells are used in large scale fermentation to produce large quantities of the cysteine protease which may be isolated or purified and used in cleaning products, such as detergents.

I. Cysteine Protease Sequences

The CP1, CP2 and CP3 polynucleotides having the sequences as shown in Figures 1A-1B, 5A-5B and 6A-6B, respectively, encode the *Bacillus subtilis* cysteine proteases CP1, CP2 and CP3. As will be understood by the skilled artisan, due to the degeneracy of the genetic code, a variety of polynucleotides can encode the *Bacillus subtilis* CP1, CP2 and CP3. The present invention encompasses all such polynucleotides.

The present invention encompasses CP1, CP2 and CP3 polynucleotide homologs encoding gram-positive microorganism cysteine proteases CP1, CP2 and CP3, respectively, which have at least 80%, or at least 90% or at least 95% identity to *B.subtilis* CP1, CP2 and CP3 as long as the homolog encodes a protein that has proteolytic activity.

Gram-positive polynucleotide homologs of *B.subtilis* CP1, CP2 or CP3 may be obtained by standard procedures known in the art from, for example, cloned DNA (*e.g.*, a DNA "library"). genomic DNA libraries, by chemical synthesis once identified, by cDNA cloning, or by the

cloning of genomic DNA, or fragments thereof, purified from a desired cell. (*See*, for example, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) A preferred source is from genomic DNA. Nucleic acid sequences derived from genomic DNA may contain regulatory regions in addition to coding regions. Whatever the source, the isolated CP1, CP2 or CP3 gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the CP1, CP2 or CP3 may be accomplished in a number of ways. For example, a *B.subtilis* CP1, CP2 or CP3 gene of the present invention or its specific RNA, or a fragment thereof, such as a probe or primer, may be isolated and labeled and then used in hybridization assays to detect a gram-positive CP1, CP2 or CP3 gene. (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. And Hogness, D., 1975, Proc. Natl. Acad. Sci. USA 72:3961). Those DNA fragments sharing substantial sequence similarity to the probe will hybridize under stringent conditions.

Accordingly, the present invention provides a method for the detection of gram-positive CP1, CP2 and CP3 polynucleotide homologs which comprises hybridizing part or all of a nucleic acid sequence of *B. subtilis* CP1, CP2 and CP3 with gram-positive microorganism nucleic acid of either genomic or cDNA origin.

Also included within the scope of the present invention are gram-positive microorganism polynucleotide sequences that are capable of hybridizing to the nucleotide sequence of *B.subtilis* CP1, CP2 or CP3 under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA) incorporated herein by reference, and confer a defined "stringency" as explained below.

"Maximum stringency" typically occurs at about Tm-5°C (5°C below the Tm of the probe); "high stringency" at about 5°C to 10°C below Tm; "intermediate stringency" at about 10°C to 20°C below Tm; and "low stringency" at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to

identify or detect identical polynucleotide sequences while an intermediate or low stringency hybridization can be used to identify or detect polynucleotide sequence homologs.

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York NY).

The process of amplification as carried out in polymerase chain reaction (PCR) technologies is described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY). A nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides from *B. subtilis* CP1, CP2 or CP3 preferably about 12 to 30 nucleotides, and more preferably about 20-25 nucleotides can be used as a probe or PCR primer.

The *B.subtilis* amino acid sequences CP1, CP2 and CP3 (shown in Figures 2, 4 and 3, respectively) were identified via a FASTA search of *Bacillus subtilis* genomic nucleic acid sequences. *B. subtilis* CP1 (YJDE) was identified by its structural homology to the cysteine protease papain having the sequence designated "papa_carpa.p". As shown in Figure 2, YJDE has the motif GXCWAF as well as the conserved catalytic residues His/Ala and Asn/Ser. CP2 (YdHS) and CP3 (PMI) were identified upon their structural homology to CP1 (YJDE). The presence of GXCWAF as well as residues His/Ala and Asn/Ser is noted in Figures 3 and 4. CP3 (PMI) was previously characterized as a possible phosphomannose isomerase, (Noramata). There has been no previous characterization of CP3 as a cysteine protease.

II. Expression Systems

The present invention provides host cells, expression methods and systems for the enhanced production and secretion of desired heterologous or homologous proteins in gram-positive microorganisms. In one embodiment, a host cell is genetically engineered to have a deletion or mutation in the gene encoding a gram-positive CP1, CP2 or CP3 such that the respective activity is deleted. In another embodiment of the present invention, a gram-positive microorganism is genetically engineered to produce a cysteine protease of the present invention.

Inactivation of a gram-positive cysteine protease in a host cell

Producing an expression host cell incapable of producing the naturally occurring cysteine protease necessitates the replacement and/or inactivation of the naturally occurring gene from the genome of the host cell. In a preferred embodiment, the mutation is a non-reverting mutation.

One method for mutating nucleic acid encoding a gram-positive cysteine protease is to clone the nucleic acid or part thereof, modify the nucleic acid by site directed

mutagenesis and reintroduce the mutated nucleic acid into the cell on a plasmid. By homologous recombination, the mutated gene may be introduced into the chromosome. In the parent host cell, the result is that the naturally occurring nucleic acid and the mutated nucleic acid are located in tandem on the chromosome. After a second recombination, the modified sequence is left in the chromosome having thereby effectively introduced the mutation into the chromosomal gene for progeny of the parent host cell.

Another method for inactivating the cysteine protease proteolytic activity is through deleting the chromosomal gene copy. In a preferred embodiment, the entire gene is deleted, the deletion occurring in such as way as to make reversion impossible. In another preferred embodiment, a partial deletion is produced, provided that the nucleic acid sequence left in the chromosome is too short for homologous recombination with a plasmid encoded cysteine protease gene. In another preferred embodiment, nucleic acid encoding the catalytic amino acid residues are deleted.

Deletion of the naturally occurring gram-positive microorganism cysteine protease can be carried out as follows. A cysteine protease gene including its 5' and 3' regions is isolated and inserted into a cloning vector. The coding region of the cysteine protease gene is deleted form the vector *in vitro*, leaving behind a sufficient amount of the 5' and 3' flanking sequences to provide for homologous recombination with the naturally occurring gene in the parent host cell. The vector is then transformed into the gram-positive host cell. The vector integrates into the chromosome via homologous recombination in the flanking regions. This method leads to a gram-positive strain in which the protease gene has been deleted.

The vector used in an integration method is preferably a plasmid. A selectable marker may be included to allow for ease of identification of desired recombinant microorgansims. Additionally, as will be appreciated by one of skill in the art, the vector is preferably one which can be selectively integrated into the chromosome. This can be achieved by introducing an inducible origin of replication, for example, a temperature sensitive origin into the plasmid. By growing the transformants at a temperature to which the origin of replication is sensitive, the replication function of the plasmid is inactivated, thereby providing a means for selection of chromosomal integrants. Integrants may be selected for growth at high temperatures in the presence of the selectable marker, such as an antibiotic. Integration mechanisms are described in WO 88/06623.

Integration by the Campbell-type mechanism can take place in the 5' flanking region of the protease gene, resulting in a protease positive strain carrying the entire plasmid vector in the chromosome in the cysteine protease locus. Since illegitimate recombination will give different results it will be necessary to determine whether the complete gene has been deleted, such as through nucleic acid sequencing or restriction maps.

Another method of inactivating the naturally occurring cysteine protease gene is to mutagenize the chromosomal gene copy by transforming a gram-positive microorganism

with oligonucleotides which are mutagenic. Alternatively, the chromosomal cysteine protease gene can be replaced with a mutant gene by homologous recombination.

The present invention encompasses host cells having deletions or mutations of a cysteine protease of the present invention as well as additional protease deletions or mutations, such as deletions or mutations in apr, npr, epr, mpr and others known to those of skill in the art. United States Patent 5,264,366 discloses *Bacillus* host cells having a deletion of apr and npr; United States Patent 5,585,253 discloses *Bacillus* host cells having a deletion of epr; Margot et al., 1996, Microbiology 142: 3437-3444 disclose host cells having a deletion in wpr and EP patent 0369817 discloses *Bacillus* host cells having a deletion of mpr.

One assay for the detection of mutants involves growing the *Bacillus* host cell on medium containing a protease substrate and measuring the appearance or lack thereof, of a zone of clearing or halo around the colonies. Host cells which have an inactive protease will exhibit little or no halo around the colonies.

III. Production of Cysteine Protease

For production of cysteine protease in a host cell, an expression vector comprising at least one copy of nucleic acid encoding a gram-positive microorganism CP1, CP2 or CP3, and preferably comprising multiple copies, is transformed into the host cell under conditions suitable for expression of the cysteine protease. In accordance with the present invention, polynucleotides which encode a gram-positive microorganism CP1, CP2 or CP3, or fragments thereof, or fusion proteins or polynucleotide homolog sequences that encode amino acid variants of *B.subtilis* CP1, CP2 or CP3, may be used to generate recombinant DNA molecules that direct their expression in host cells. In a preferred embodiment, the gram-positive host cell belongs to the genus *Bacillus*. In another preferred embodiment, the gram positive host cell is *B. subtilis*.

As will be understood by those of skill in the art, it may be advantageous to produce polynucleotide sequences possessing non-naturally occurring codons. Codons preferred by a particular gram-positive host cell (Murray E et al (1989) Nuc Acids Res 17:477-508) can be selected, for example, to increase the rate of expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

Altered CP1, CP2 or CP3 polynucleotide sequences which may be used in accordance with the invention include deletions, insertions or substitutions of different nucleotide residues resulting in a polynucleotide that encodes the same or a functionally equivalent CP1, CP2 or CP3 homolog, respectively. As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

As used herein an "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring CP1, CP3 or CP3.

As used herein "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

The encoded protein may also show deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally CP1, CP2 or CP3 variant. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the variant retains the ability to modulate secretion. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine, phenylalanine, and tyrosine.

The CP1, CP2 or CP3 polynucleotides of the present invention may be engineered in order to modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, eg, site-directed mutagenesis to insert new restriction sites, to alter glycosylation patterns or to change codon preference, for example.

In one embodiment of the present invention, a gram-positive microorganism CP1, CP2 or CP3 polynucleotide may be ligated to a heterologous sequence to encode a fusion protein. A fusion protein may also be engineered to contain a cleavage site located between the cysteine protease nucleotide sequence and the heterologous protein sequence, so that the cysteine protease may be cleaved and purified away from the heterologous moiety.

IV. Vector Sequences

Expression vectors used in expressing the cysteine proteases of the present invention in gram-positive microorganisms comprise at least one promoter associated with a cysteine protease selected from the group consisting of CP1, CP2 and CP3, which promoter is functional in the host cell. In one embodiment of the present invention, the promoter is the wild-type promoter for the selected cysteine protease and in another embodiment of the present invention, the promoter is heterologous to the cysteine protease, but still functional in the host cell. In one preferred embodiment of the present invention, nucleic acid encoding the cysteine protease is stably integrated into the microorganism genome.

In a preferred embodiment, the expression vector contains a multiple cloning site cassette which preferably comprises at least one restriction endonuclease site unique to the vector, to facilitate ease of nucleic acid manipulation. In a preferred embodiment, the vector also comprises one or more selectable markers. As used herein, the term selectable

marker refers to a gene capable of expression in the gram-positive host which allows for ease of selection of those hosts containing the vector. Examples of such selectable markers include but are not limited to antibiotics, such as, erythromycin, actinomycin, chloramphenicol and tetracycline.

V. Transformation

A variety of host cells can be used for the production of CP1, CP2 and CP3 including bacterial, fungal, mammalian and insects cells. General transformation procedures are taught in Current Protocols In Molecular Biology (vol. 1, edited by Ausubel et al., John Wiley & Sons, Inc. 1987, Chapter 9) and include calcium phosphate methods, transformation using DEAE-Dextran and electroporation. Plant transformation methods are taught in Rodriquez (WO 95/14099, published 26 May 1995).

In a preferred embodiment, the host cell is a gram-positive microorganism and in another preferred embodiment, the host cell is *Bacillus*. In one embodiment of the present invention, nucleic acid encoding one or more cysteine protease(s) of the present invention is introduced into a host cell via an expression vector capable of replicating within the *Bacillus* host cell. Suitable replicating plasmids for *Bacillus* are described in Molecular Biological Methods for *Bacillus*, Ed. Harwood and Cutting, John Wiley & Sons, 1990, hereby expressly incorporated by reference; see chapter 3 on plasmids. Suitable replicating plasmids for *B. subtilis* are listed on page 92.

In another embodiment, nucleic acid encoding a cysteine protease(s) of the present invention is stably integrated into the microorganism genome. Preferred host cells are gram-positive host cells. Another preferred host is *Bacillus*. Another preferred host is *Bacillus subtilis*. Several strategies have been described in the literature for the direct cloning of DNA in *Bacillus*. Plasmid marker rescue transformation involves the uptake of a donor plasmid by competent cells carrying a partially homologous resident plasmid (Contente et al., Plasmid 2:555-571 (1979); Haima et al., Mol. Gen. Genet. 223:185-191 (1990); Weinrauch et al., J. Bacteriol. 154(3):1077-1087 (1983); and Weinrauch et al., J. Bacteriol. 169(3):1205-1211 (1987)). The incoming donor plasmid recombines with the homologous region of the resident "helper" plasmid in a process that mimics chromosomal transformation.

Transformation by protoplast transformation is described for *B. subtilis* in Chang and Cohen, (1979) Mol. Gen. Genet 168:111-115; for B.megaterium in Vorobjeva et al., (1980) FEMS Microbiol. Letters 7:261-263; for B. amyloliquefaciens in Smith et al., (1986) Appl. and Env. Microbiol. 51:634; for B.thuringiensis in Fisher et al., (1981) Arch. Microbiol. 139:213-217; for B.sphaericus in McDonald (1984) J. Gen. Microbiol. 130:203; and B.larvae in Bakhiet et al., (1985) 49:577. Mann et al., (1986, Current Microbiol. 13:131-135) report on transformation of *Bacillus* protoplasts and Holubova, (1985) Folia Microbiol. 30:97) disclose methods for introducing DNA into protoplasts using DNA containing liposomes.

VI. Identification of Transformants

Whether a host cell has been transformed with a mutated or a naturally occurring gene encoding a gram-positive CP1, CP2 or CP3, detection of the presence/absence of marker gene expression can suggests whether the gene of interest is present However, its expression should be confirmed. For example, if the nucleic acid encoding a cysteine protease is inserted within a marker gene sequence, recombinant cells containing the insert can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with nucleic acid encoding the cysteine protease under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the cysteine protease as well.

Alternatively, host cells which contain the coding sequence for a cysteine protease and express the protein may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

The presence of the cysteine polynucleotide sequence can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes, portions or fragments of *B.subtilis* CP1, CP2 or CP3.

VII. Assay of Protease Activity

There are various assays known to those of skill in the art for detecting and measuring protease activity. There are assays based upon the release of acid-soluble peptides from casein or hemoglobin measured as absorbance at 280 nm or colorimetrically using the Folin method (Bergmeyer, et al., 1984, Methods of Enzymatic Analysis vol. 5, Peptidases, Proteinases and their Inhibitors, Verlag Chemie, Weinheim). Other assays involve the solubilization of chromogenic substrates (Ward, 1983, Proteinases, in Microbial Enzymes and Biotechnology (W.M. Fogarty, ed.), Applied Science, London, pp. 251-317).

VIII. Secretion of Recombinant Proteins

Means for determining the levels of secretion of a heterologous or homologous protein in a gram-positive host cell and detecting secreted proteins include, using either polyclonal or monoclonal antibodies specific for the protein. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). These and other assays are described, among other places, in Hampton R et al (1990, Serological Methods, a Laboratory Manual, APS Press, St Paul MN) and Maddox DE et al (1983, J Exp Med 158:1211).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting specific polynucleotide sequences include

oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the nucleotide sequence, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides.

A number of companies such as Pharmacia Biotech (Piscataway NJ), Promega (Madison WI), and US Biochemical Corp (Cleveland OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241. Also, recombinant immunoglobulins may be produced as shown in US Patent No. 4,816,567 and incorporated herein by reference.

IX. Purification of Proteins

Gram positive host cells transformed with polynucleotide sequences encoding heterologous or homologous protein may be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The protein produced by a recombinant gram-positive host cell comprising a mutation or deletion of the cysteine protease activity will be secreted into the culture media. Other recombinant constructions may join the heterologous or homologous polynucleotide sequences to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll DJ et al (1993) DNA Cell Biol 12:441-53).

Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath J (1992) Protein Expr Purif 3:263-281), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego CA) between the purification domain and the heterologous protein can be used to facilitate purification.

X. Uses of The Present Invention

CP1, CP2 and CP3 and Genetically Engineered Host Cells

The present invention provides genetically engineered host cells comprising preferably non-revertable mutations or deletions in the naturally occurring gene encoding CP1, CP2 or CP3 such that the proteolytic activity is diminished or deleted altogether. The host cell may contain additional protease deletions, such as deletions of the mature subtilisn protease and/or mature neutral protease disclosed in United States Patent No. 5,264,366.

In a preferred embodiment, the host cell is further genetically engineered to produce a desired protein or polypeptide. In a preferred embodiment the host cell is a *Bacillus*. In another preferred embodiment, the host cell is a *Bacillus subtilis*.

In an alternative embodiment, a host cell is genetically engineered to produce a gram-positive CP1, CP2 or CP3. In a preferred embodiment, the host cell is grown under large scale fermentation conditions, the CP1, CP2 or CP3 is isolated and/or purified and used in cleaning compositions such as detergents. Detergent formulations are disclosed in WO 95/10615. A cysteine protease of the present invention can be useful in formulating various cleaning compositions. A number of known compounds are suitable surfactants useful in compositions comprising the cysteine protease of the invention. These include nonionic, anionic, cationic, anionic or zwitterionic detergents, as disclosed in US 4,404,128 and US 4,261,868. A suitable detergent formulation is that described in Example 7 of US Patent 5,204,015. The art is familiar with the different formulations which can be used as cleaning compositions. In addition, a cysteine protease of the present invention can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide hydrolysis, waste treatment, textile applications, as fusion-cleavage enzymes in protein production, etc. A cysteine protease may provide enhanced performance in a detergent composition (as compared to another detergent protease). As used herein, enhanced performance in a detergent is defined as increasing cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle.

A cysteine protease of the present invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about .01 to about 5% (preferably .1% to .5%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

The addition of a cysteine protease to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the described cysteine protease denaturing temperature. In addition, a cysteine protease can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

One aspect of the invention is a composition for the treatment of a textile that includes a cysteine protease of the present invention. The composition can be used to treat for example silk or wool as described in publications such as RD 216,034; EP 134,267; US 4,533,359; and EP 344,259.

Proteases can be included in animal feed such as part of animal feed additives as described in, for example, US 5,612,055; US 5,314,692; and US 5,147,642.

CP1, CP2 and CP3 Polynucleotides

A *B.subtlis* polynucleotide, or any part thereof, provides the basis for detecting the presence of gram-positive microorganism polynucleotide homologs through hybridization techniques and PCR technology.

Accordingly, one aspect of the present invention is to provide for nucleic acid hybridization and PCR probes which can be used to detect polynucleotide sequences, including genomic and cDNA sequences, encoding gram-positive CP1, CP2 or CP3 or portions thereof.

The manner and method of carrying out the present invention may be more fully understood by those of skill in the art by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto

Example I

Preparation of a Genomic library

The following example illustrates the preparation of a *Bacillus* genomic library.

Genomic DNA from *Bacillus* cells is prepared as taught in Current Protocols In Molecular Biology vol. 1, edited by Ausubel et al., John Wiley & Sons, Inc. 1987, chapter 2. 4.1. Generally, *Bacillus* cells from a saturated liquid culture are lysed and the proteins removed by digestion with proteinase K. Cell wall debris, polysaccharides, and remaining proteins are removed by selective precipitation with CTAB, and high molecular weight genomic DNA is recovered from the resulting supernatant by isopropanol precipitation. If exceptionally clean genomic DNA is desired. an additional step of purifying the *Bacillus* genomic DNA on a cesium chloride gradient is added.

After obtaining purified genomic DNA, the DNA is subjected to Sau3A digestion. Sau3A recognizes the 4 base pair site GATC and generates fragments compatible with several convenient phage lambda and cosmid vectors. The DNA is subjected to partial digestion to increase the chance of obtaining random fragments.

The partially digested *Bacillus* genomic DNA is subjected to size fractionation on a 1% agarose gel prior to cloning into a vector. Alternatively, size fractionation on a sucrose gradient can be used. The genomic DNA obtained from the size fractionation step is purified away from the agarose and ligated into a cloning vector appropriate for use in a host cell and transformed into the host cell.

Example II

Detection of gram-postive microorganisms

The following example describes the detection of gram-positive microorganism CP1. The same procedures can be used to detect CP2 and CP3.

DNA derived from a gram-positive microorganism is prepared according to the methods disclosed in Current Protocols in Molecular Biology, Chap. 2 or 3. The nucleic acid is subjected to hybridization and/or PCR amplification with a probe or primer derived from CP1. A preferred probe comprises the nucleic acid section containing the conserved motif GXCWAF.

The nucleic acid probe is labeled by combining 50 pmol of the nucleic acid and 250 mCi of [gamma ^{32}P] adenosine triphosphate (Amersham, Chicago IL) and T4 polynucleotide kinase (DuPont NEN®, Boston MA). The labeled probe is purified with Sephadex G-25 super fine resin column (Pharmacia). A portion containing 10^7 counts per minute of each is used in a typical membrane based hybridization analysis of nucleic acid sample of either genomic or cDNA origin.

The DNA sample which has been subjected to restriction endonuclease digestion is fractionated on a 0.7 percent agarose gel and transferred to nylon membranes (Nytran Plus, Schleicher & Schuell, Durham NH). Hybridization is carried out for 16 hours at 40 degrees C. To remove nonspecific signals, blots are sequentially washed at room temperature under increasingly stringent conditions up to 0.1 x saline sodium citrate and 0.5% sodium dodecyl sulfate. The blots are exposed to film for several hours, the film developed and hybridization patterns are compared visually to detect polynucleotide homologs of *B.subtilis* CP1. The homologs are subjected to confirmatory nucleic acid sequencing. Methods for nucleic acid sequencing are well known in the art. Conventional enzymatic methods employ DNA polymerase Klenow fragment, SEQUENASE® (US Biochemical Corp, Cleveland, OH) or Taq polymerase to extend DNA chains from an oligonucleotide primer annealed to the DNA template of interest.

Various other examples and modifications of the foregoing description and examples will be apparent to a person skilled in the art after reading the disclosure without departing from the spirit and scope of the invention, and it is intended that all such examples or odifications be included within the scope of the appended claims. All publications and patents referenced herein are hereby incorporated by reference in their entirety.

CLAIMS

1. A gram-positive microorganism having a mutation or deletion of part or all of the gene encoding CP1 said mutation or deletion resulting in the inactivation of the CP1 proteolytic activity.

2. A gram-positive microorganism having a mutation or deletion of part or all of the gene encoding CP2 said mutation or deletion resulting in the inactivation of the CP2 proteolytic activity.

3. A gram-positive microorganism having a mutation or deletion of part or all of the gene encoding CP3 said mutation or deletion resulting in the inactivation of the CP3 proteolytic activity.

4. The gram-positive microorganism according to Claims 1, 2 or 3 that is a member of the family *Bacillus*.

5. The microorganism according to Claim 4 wherein the member is selected from the group consisting of *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus and Bacillus thuringiensis*.

6. The microorganism of Claim 1, 2 or 3 wherein said microorganism is capable of expressing a heterologous protein.

7. The microorganism of Claim 6 wherein said heterologous protein is selected from the group consisting of hormone, enzyme, growth factor and cytokine.

8. The microorganism of Claim 7 wherein said heterologous protein is an enzyme.

9. The microorganism of Claim 8 wherein said enzyme is selected from the group consisting of a proteases, carbohydrases, and lipases; isomerases such as racemases, epimerases, tautomerases, or mutases; transferases, kinases and phophatases.

10. A cleaning composition comprising at least one cysteine protease selected from the group consisting of CP1, CP2 and CP3.

11. An expression vector comprising nucleic acid encoding a cysteine protease selected from the group consisting of CP1, CP2 and CP3.

12. A host cell comprising an expression vector according to Claim 11.

13. A method for the production of a heterologous protein in a *Bacillus* host cell comprising the steps of

(a) obtaining a *Bacillus* host cell comprising nucleic acid encoding said heterologous protein wherein said host cell contains a mutation or deletion in at least one of the genes encoding cysteine protease 1, cysteine protease 2 and cysteine protease 3; and

(b) growing said *Bacillus* host cell under conditions suitable for the expression of said heterologous protein.

14. The method of Claim 13 wherein said *Bacillus* cell is selected from the group consisting of *Bacillus subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus and Bacillus thuringiensis.*

15. The method of Claim 13 wherein said Bacillus host cell further comprises a mutation or deletion in at least one of the genes encoding apr, npr, epr, wpr and mrp.

16. A gram-positive microorganism having at mutation or deletion in at least one of the genes encoding a cysteine protease selected from the group consisting of CP1, CP2 and CP3.

17. The microorganism of Claim 16 further comprising a mutation or deletion in at least one of the genes encoding apr, npr, epr, wpr and mrp.

```
          10                  30
atgacgactgaaccgttatttttcaagcctgttttcaaagaaagaatt
M  T  T  E  P  L  F  F  K  P  V  F  K  E  R  I

50                  70                  90
tggggcgggaccgctttagctgattttggctataccattccgtcacaa
W  G  G  T  A  L  A  D  F  G  Y  T  I  P  S  Q

          110                 130
cgaacaggggagtgctgggcttttgccgcgcatcaaaatggtcaaagc
R  T  G  E  C  W  A  F  A  A  H  Q  N  G  Q  S

   150                 170                 190
gttgttcaaaacggaatgtataaggggttcacgctcagcgaattatgg
V  V  Q  N  G  M  Y  K  G  F  T  L  S  E  L  W

                210                 230
gaacatcacagacatttattcggacagcttgaaggggaccgtttccct
E  H  H  R  H  L  F  G  Q  L  E  G  D  R  F  P

        250                 270                 2
ctgcttacaaaaatattagatgctgaccaggacttatctgttcaggtg
L  L  T  K  I  L  D  A  D  Q  D  L  S  V  Q  V

90                  310                 330
catccgaatgatgaatatgccaacatacatgaaaacggtgagcttgga
H  P  N  D  E  Y  A  N  I  H  E  N  G  E  L  G

          350                 370
aaaacagaatgctggtacattattgattgccaaaaagatgccgagatt
K  T  E  C  W  Y  I  I  D  C  Q  K  D  A  E  I

   390                 410                 430
atttatggccacaatgcaacaacaaaggaagaactaactaccatgata
I  Y  G  H  N  A  T  T  K  E  E  L  T  T  M  I

                450                 470
gagcgtggagaatgggatgagctcttgcgccgtgtaaaggtaaagccg
E  R  G  E  W  D  E  L  L  R  R  V  K  V  K  P

        490                 510                 5
ggggatttttctatgtgccaagcggtactgttcatgcgattggaaaa
G  D  F  F  Y  V  P  S  G  T  V  H  A  I  G  K

30                  550                 570
ggaattcttgctttggagacgcagcagaactcagacacaacctacaga
G  I  L  A  L  E  T  Q  Q  N  S  D  T  T  Y  R
```

FIG._1A

```
            590                   610
ttatatgattatgaccgaaaagatgcagaaggcaagctgcgcgagctt
L  Y  D  Y  D  R  K  D  A  E  G  K  L  R  E  L

   630                   650                   670
catctgaaaaagagcattgaagtgatagaggtcccgtctattccagaa
H  L  K  K  S  I  E  V  I  E  V  P  S  I  P  E

                690                   710
cggcatacagttcaccatgaacaaattgaggatttgcttacaacgaca
R  H  T  V  H  H  E  Q  I  E  D  L  L  T  T  T

       730                   750                   7
ttgattgaatgcgcttacttttcggtggggaaatggaacttatcagga
L  I  E  C  A  Y  F  S  V  G  K  W  N  L  S  G

70                   790                   810
tcagcaagcttaaagcagcaaaaaccattccttcttatcagtgtgatt
S  A  S  L  K  Q  Q  K  P  F  L  L  I  S  V  I

            830                   850
gaaggggagggccgtatgatctctggtgagtatgtctatcctttcaaa
E  G  E  G  R  M  I  S  G  E  Y  V  Y  P  F  K

   870                   890                   910
aaaggagatcatatgttgctgccttacggtcttggagaatttaaactc
K  G  D  H  M  L  L  P  Y  G  L  G  E  F  K  L

                930
gaaggatatgcagaatgtatcgtctcccatctg
E  G  Y  A  E  C  I  V  S  H  L
```

FIG._1B

papa_carpa.p VLNDGDVNIPEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVTIEGIIKIRTGNLNEYSE
YJDE PLFFKPVFKERIWGGTALADFGYTIPSQRTGECWAFAAHQNGQSVVQ--NGMYKGFTL
papa_carpa.p LLDCDRRSYGCNGG--YPWSALQLVAQYGIHYRNTYPYEGVQRYCRSREKGPYAAKTD
YJDE LWEHHRHLFGQLEGDRFPLLTKILDADQDLSVQ-VHPND---EYANIHENGELG-KTE
papa_carpa.p RQVQPYNEGALLY---SIANQPVSVVLEAAGKDFQLYR----GGIFVGPCGNKVDHA
YJDE YIIDCQKDAEIIYGHNATTKEELTTMIERGEWDELLRRVKVKPGDFFYVPSGT-----
papa_carpa.p AVGYGPNYILIKNSWGTGWGENGYIRIKRGTGNSYGVCGLYTSSFYPVKN
YJDE AIGKGILALETQQNSDTTYRLYDYDRKDAEGKLRELHLKKSIEVIEVPSIPERHTVHH
130 140 150 160 170 180
10 20 30 40 50 60
190 200 210 220 230 240
70 80 90 100 110
250 260 270 280 290
120 130 140 150 160 170
300 310 320 330 340
180 190 200 210 220 230

FIG._2

```
                10        20        30        40        50
59 yjde.pep     MTTEPLFFKPVFKERIWGGTALAD-FGYTIPSQRTGECWAFAAHQNGQSVVQNGMYKG
FT              ||  |:|: |||||:|||||||| | |||:|||: |||||||:::|| :| |:| || |||
   PMI          MTQSPIFLTPVFKEKIWGGTALRDRFGYSIPSESTGECWAISAHPKGPSTVANGPYKG
KT                    10        20        30        40        50
60                60        70        80        90        100       110       1
19 yjde.pep     LSELWEHHRHLFGQLEGDRFPLLTKILDADQDLSVQVHPNDEYANIHENGELGKTECW
YI              | |||||:||::|| :|||||||||||:||: :| |::|||:| ||: :|:|||||||||
   PMI          LIELWEEHREVFGGVEGDRFPLLTKLLDVKEDTSIKVHPDDYYAGENEEGELGKTECW
YI                    70        80        90        100       110       1
20                120       130       140       150       160       170       1
79 yjde.pep     IDCQKDAEIIYGHNATTKEELTMIERGEWDELLRRVKVKPGDFFYVPSGTVHAIGKG
IL              |||:::|||||||||:| :| ||:|||: |:|: ||||:|:|||||:|||||:||: ||
   PMI          IDCKENAEIIYGHTARSKTELVTMINSGDWEGLLRRIKIKPGDFYYVPSGTLHALCKG
AL                    130       140       150       160       170       1
80
```

FIG._3A

```
              180        190       200       210       220       230       2
39
yjde.pep      ALETQQNSDTTYRLYDYDRKDAEGKLRELHLKKSIEVIEVPSIPERHTVHHEQIEDLL
TT
              :||||||||:|||:||||| |::|: ||||: |:::: || :         |: : :
PMI           VLETQQNSDATYRVYDYDRLDSNGSPRELHFAKAVNAATVPHVDGYIDESTESRKGIT
IK
                     190       200       210       220       230       2
40

             240       250       260       270       280       290       2
99
yjde.pep      TLIECAYFSVGKWNLSGSASLKQQKPFLLISVIEGEGRMISGEYVYPFKKGDHMLLPY
GL
              |:::  |||| ||:::| | : |:: ||: ||||| | :   : : |:||||||::||
 :
PMI           TFVQGEYFSVYKWDINGEAEMAQDESFLICSVIEGSGLLKYEDKTCPLKKGDHFILPA
QM
                     250       260       270       280       290       3
00

             300       310
yjde.pep      GEFKLEGYAECIVSHL
               :| ::|    ||||:
PMI           PDFTIKGTCTLIVSHI
                     310
```

FIG._3B

```
                10        20        30        40        50
59 yjde.pep     MTTEPLFFKPVFKERIWGGTALAD-FGYTIPSQRTGECWAFAAHQNGQSVVQNGMYKG
FT                |:||||::|||||||:|||||  |  |  |||:|||||:|||||||  :||  :|:|  |:||    |
 1 YDHS         MTHPLFLEPVFKERLWGGTKLRDAFGYAIPSQKTGECWAVSAHAHGSSSVKNGPLAG
KT                      10        20        30        40        50

                60        70        80        90        100       110       1
19 yjde.pep     LSELWEHHRHLFGQLEGDRFPLLTKILDADQDLSVQVHPNDEYANIHENGELGKTECW
YI                |::::|:  |  ::||  :|  ||||:|:||||::|||||||||:|:|||::|||||:||||||||
11 YDHS         LDQVWKDHPEIFGFPDGKVFPLLVKLLDANMDLSVQVHPDDDYAKLHENGDLGKTECW
YI                      70        80        90        100       110

                120       130       140       150       160       170       1
79 yjde.pep     IDCQKDAEIIYGHNATTKEELTMIERGEWDELLRRVKVKPGDFFYVPSGTVHAIGKG
IL                |||:  |||:|  ||:|:||||:    ||  |:|:  ||||:|:|||||||||||||:||:  ||
 1 YDHS         IDCKDDAELILGHHASTKEEFKQRIESGDWNGLLRRIKIKPGDFFYVPSGTLHALCKG
TL                120       130       140       150       160       170
```

FIG._4A

```
            180       190       200       210       220       230       2
39                ↓↓
yjde.pep    ALETQQNSDTTYRLYDYDRKDAEGKLRELHLKKSIEVIEVPSIPERHTVHHEQIEDLL
TT
            :|| |||||||||:||||| : :|: | ||::|::||| :| | : || : ::: :
YDHS        VLEIQQNSDTTYRVYDYDRCNDQGQKRTLHIEKAMEVITIPHIDKVHTPEVKEVGNAE
II                ↑↑
            180       190       200       210       220       230

            240       250       260       270       280       290       2
99
yjde.pep    TLIECAYFSVGKWNLSGSASLKQQKPFLLISVIEGEGRMISGEYVYPFKKGDHMLLPY
GL
            : ::  |||| ||::|| |:: : : :|| ||: | ||:|::   |  : |:|::||
 :
YDHS        VYVQSDYFSVYKWKISGRAAFPSYQTYLLGSVLSGSGRIINNGIQYECNAGSHFILPA
HF
            240       250       260       270       280       290

            300       310
yjde.pep    GEFKLEGYAECIVSHL
            ||| :||  | ::||
YDHS        GEFTIEGTCEFMISHP
            300       310
```

FIG._4B

```
          10                  30
atgacgcatccattatttttagagcctgtctttaaagaaagactatgg
M  T  H  P  L  F  L  E  P  V  F  K  E  R  L  W

50                  70                  90
ggagggacgaagcttcgtgacgcttttggctacgcaataccctcacaa
G  G  T  K  L  R  D  A  F  G  Y  A  I  P  S  Q

          110                 130
aaaacaggtgagtgctgggccgtttctgcacatgcccatggctcgtcg
K  T  G  E  C  W  A  V  S  A  H  A  H  G  S  S

   150                 170                 190
tctgtaaaaaatggcccgctggcaggaaagacacttgatcaagtatgg
S  V  K  N  G  P  L  A  G  K  T  L  D  Q  V  W

               210                 230
aaagatcatccagagatattcgggtttccggatggtaaggtgtttccg
K  D  H  P  E  I  F  G  F  P  D  G  K  V  F  P

       250                 270                 2
ctgctggtaaagctgctggacgccaatatggatctctccgtgcaagtc
L  L  V  K  L  L  D  A  N  M  D  L  S  V  Q  V

90                  310                 330
catcctgatgatgattatgcaaaactgcacgaaaatggcgaccttggt
H  P  D  D  D  Y  A  K  L  H  E  N  G  D  L  G

          350                 370
aaaacggagtgctggtatatcattgattgcaaagatgacgccgaacta
K  T  E  C  W  Y  I  I  D  C  K  D  D  A  E  L

   390                 410                 430
attttgggacatcatgcaagcacaaaggaagagttcaaacaacgaata
I  L  G  H  H  A  S  T  K  E  E  F  K  Q  R  I

               450                 470
gaaagcggtgattggaacgggctgctgaggcgaatcaaaatcaagcca
E  S  G  D  W  N  G  L  L  R  R  I  K  I  K  P

       490                 510                 5
ggagatttcttttatgtgccaagcggtacactccatgctttatgtaag
G  D  F  F  Y  V  P  S  G  T  L  H  A  L  C  K

30                  550                 570
ggaacccttgtccttgaaatccagcaaaactctgatacaacatatcgc
G  T  L  V  L  E  I  Q  Q  N  S  D  T  T  Y  R
```

FIG._5A

```
            590                   610
gtatacgattatgaccgctgtaatgaccagggccaaaaaagaactctt
V  Y  D  Y  D  R  C  N  D  Q  G  Q  K  R  T  L

   630                   650                   670
catatagaaaaagccatggaagtcataacgataccgcatatcgataaa
H  I  E  K  A  M  E  V  I  T  I  P  H  I  D  K

                  690                   710
gtgcatacaccggaagtaaaagaagttggtaacgctgagatcattgtt
V  H  T  P  E  V  K  E  V  G  N  A  E  I  I  V

        730                   750                   7
tatgtgcaatcagattatttctcagtgtacaaatggaagattagcggc
Y  V  Q  S  D  Y  F  S  V  Y  K  W  K  I  S  G

70                  790                   810
cgagctgcttttccttcatatcaaacctatttgctggggagtgttctg
R  A  A  F  P  S  Y  Q  T  Y  L  L  G  S  V  L

            830                   850
agcggatcaggacgaatcataaataatggtattcagtatgaatgcaat
S  G  S  G  R  I  I  N  N  G  I  Q  Y  E  C  N

   870                   890                   910
gcaggctcacactttattctgcctgcgcattttggagaatttacaata
A  G  S  H  F  I  L  P  A  H  F  G  E  F  T  I

                  930
gaaggaacatgtgaattcatgatatctcatcct
E  G  T  C  E  F  M  I  S  H  P
```

FIG._5B

```
          10                  30
atgacgcaatcaccgatttttctaacgcctgtgtttaaagaaaaaatc
M  T  Q  S  P  I  F  L  T  P  V  F  K  E  K  I

50                  70                  90
tggggcggaaccgctttacgagatagatttggatacagtattccttca
W  G  G  T  A  L  R  D  R  F  G  Y  S  I  P  S

            110                 130
gaatcaacgggggaatgctgggccatttccgctcatccaaaaggaccg
E  S  T  G  E  C  W  A  I  S  A  H  P  K  G  P

   150                 170                 190
agcactgttgcaaatggcccgtataaaggaaagacattgatcgagctt
S  T  V  A  N  G  P  Y  K  G  K  T  L  I  E  L

               210                 230
tgggaagagcaccgtgaagtattcggcggcgtagaggggatcggttt
W  E  E  H  R  E  V  F  G  G  V  E  G  D  R  F

        250                 270                 2
ccgcttctgacaaagctgctggatgtgaaggaagatacgtcaattaaa
P  L  L  T  K  L  L  D  V  K  E  D  T  S  I  K

90                  310                 330
gttcaccctgatgattactatgccggagaaaacgaagagggagaactc
V  H  P  D  D  Y  Y  A  G  E  N  E  E  G  E  L

            350                 370
ggcaagacggaatgctggtacattatcgactgtaaggaaaacgcagaa
G  K  T  E  C  W  Y  I  I  D  C  K  E  N  A  E

   390                 410                 430
atcatttacgggcatacggcccgctcaaaaaccgaacttgtcacaatg
I  I  Y  G  H  T  A  R  S  K  T  E  L  V  T  M

               450                 470
atcaacagcggtgactgggagggcctgctgcgaagaatcaaaattaaa
I  N  S  G  D  W  E  G  L  L  R  R  I  K  I  K

        490                 510                 5
ccgggtgatttctattatgtgccgagcggaacgctgcacgcattgtgc
P  G  D  F  Y  Y  V  P  S  G  T  L  H  A  L  C

30                  550                 570
aaggggccccttgttttagagactcagcaaaattcagatgccacatac
K  G  A  L  V  L  E  T  Q  Q  N  S  D  A  T  Y
```

FIG._6A

```
              590                   610
cgggtgtacgattatgaccgtcttgatagcaacggaagtccgagagag
R  V  Y  D  Y  D  R  L  D  S  N  G  S  P  R  E

   630                   650                   670
cttcattttgccaaagcggtcaatgccgccacggttccccatgtggac
L  H  F  A  K  A  V  N  A  A  T  V  P  H  V  D

                690                   710
gggtatatagatgaatcgacagaatcaagaaaaggaataaccattaaa
G  Y  I  D  E  S  T  E  S  R  K  G  I  T  I  K

        730                   750                   7
acatttgtccaaggggaatatttttcggtttataaatgggacatcaat
T  F  V  Q  G  E  Y  F  S  V  Y  K  W  D  I  N

70                    790                   810
ggcgaagctgaaatggctcaggatgaatcctttctgatttgcagcgtg
G  E  A  E  M  A  Q  D  E  S  F  L  I  C  S  V

             830                   850
atagaaggaagcggtttgctcaagtatgaggacaaaacatgtccgctc
I  E  G  S  G  L  L  K  Y  E  D  K  T  C  P  L

   870                   890                   910
aaaaaaggtgatcactttatttgccggctcaaatgcccgattttacg
K  K  G  D  H  F  I  L  P  A  Q  M  P  D  F  T

                930
ataaaaggaacttgtacccttatcgtgtctcatatt
I  K  G  T  C  T  L  I  V  S  H  I
```

FIG._6B

DECLARATION
AND POWER OF ATTORNEY

AS A BELOW NAMED INVENTOR, I HEREBY DECLARE THAT:

MY RESIDENCE, POST OFFICE ADDRESS AND CITIZENSHIP ARE AS STATED BELOW NEXT TO MY NAME. I BELIEVE I AM THE ORIGINAL, FIRST AND SOLE INVENTOR (IF ONLY ONE NAME IS LISTED BELOW) OR AN ORIGINAL, FIRST AND JOINT INVENTOR (IF PLURAL NAMES ARE LISTED BELOW) OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED **PROTEASES FROM GRAM-POSITIVE ORGANISMS** THE SPECIFICATION OF WHICH

CHECK ONE:
XX IS ATTACHED HERETO
WAS FILED ON _____ AS APPLICATION SERIAL NO. _____ AND WAS AMENDED ON _____.

I HEREBY STATE THAT I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE. I ACKNOWLEDGE THE DUTY TO DISCLOSE INFORMATION WHICH IS MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS §1.56.

I HEREBY CLAIM FOREIGN PRIORITY BENEFITS UNDER TITLE 35, UNITED STATES CODE §119, OF ANY FOREIGN APPLICATION(S) FOR PATENT OR INVENTOR'S CERTIFICATE LISTED BELOW AND HAVE ALSO IDENTIFIED BELOW ANY FOREIGN APPLICATION FOR PATENT OR INVENTOR'S CERTIFICATE HAVING A FILING DATE BEFORE THAT OF THE APPLICATION ON WHICH PRIORITY IS CLAIMED.

APPLICATION NUMBER	COUNTRY	DATE OF FILING	PRIORITY CLAIMED YES	NO

I HEREBY CLAIM THE BENEFIT UNDER TITLE 35, UNITED STATES CODE §120, OF ANY UNITED STATES APPLICATION(S) OR PCT INTERNATIONAL APPLICATION(S) DESIGNATING THE UNITED STATES OF AMERICA THAT IS LISTED BELOW AND, INSOFAR AS THE SUBJECT MATTER OF EACH OF THE CLAIMS OF THIS APPLICATION IS NOT DISCLOSED IN THE PRIOR UNITED STATES APPLICATION IN THE MANNER PROVIDED BY THE FIRST PARAGRAPH OF TITLE 35, UNITED STATES CODE §112, I ACKNOWLEDGE THE DUTY TO DISCLOSE MATERIAL INFORMATION AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS §1.56(A) WHICH OCCURRED BETWEEN THE FILING DATE OF THE PRIOR APPLICATION AND THE NATIONAL OR PCT INTERNATIONAL FILING DATE OF THIS APPLICATION.

APPLICATION NUMBER	DATE OF FILING	STATUS - PATENTED, PENDING OR ABANDONED
PCT/US98/14529	14 JULY 1998	PENDING
EP 97305227.7	15 JULY 1997	ABANDONED

POWER OF ATTORNEY: AS A NAMED INVENTOR I HEREBY APPOINT AS MY ATTORNEY(S) WITH FULL POWER OF SUBSTITUTION AND REVOCATION, TO PROSECUTE THIS APPLICATION AND TRANSACT ALL BUSINESS IN THE PATENT AND TRADEMARK OFFICE CONNECTED THEREWITH:

(4)

MARGARET A. HORN, REG. NO. ~~33,401;~~
CHRISTOPHER L. STONE, REG. NO. 35,696
JEFFERY D. FRAZIER, REG. NO. 34,601
SUSAN FARIS, REG. NO. 41,739

SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO:
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201

FULL NAME OF INVENTOR	FULL FIRST NAME DAVID	INITIAL A.	LAST NAME ESTELL		
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POST OFFICE ADDRESS	POST OFFICE ADDRESS 248 WOODBRIDGE CIRCLE,	CITY SAN MATEO		STATE OR COUNTRY CALIFORNIA	ZIP CODE 94403

I FURTHER DECLARE THAT ALL STATEMENTS MADE HEREIN OF MY OWN KNOWLEDGE ARE TRUE AND THAT ALL STATEMENTS MADE ON INFORMATION AND BELIEF ARE BELIEVED TO BE TRUE; AND FURTHER THAT THESE STATEMENTS WERE MADE WITH THE KNOWLEDGE THAT WILLFUL FALSE STATEMENTS AND THE LIKE SO MADE ARE PUNISHABLE BY FINE OR IMPRISONMENT, OR BOTH, UNDER SECTION 1001 OF TITLE 18 OF THE UNITED STATES CODE , AND THAT SUCH WILLFUL FALSE STATEMENTS MAY JEOPARDIZE THE VALIDITY OF THE APPLICATION OR ANY PATENT ISSUING THEREON.

SIGNATURE OF INVENTOR **201**	
DATE January 5, 2000	